FEBS Letters 572 (2004) 238–244 FEBS 28694

ERα and STAT5a cross-talk: interaction through C-terminal portions of the proteins decreases STAT5a phosphorylation, nuclear translocation and DNA-binding

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Received 3 June 2004; revised 19 June 2004; accepted 23 June 2004

Available online 26 July 2004

Edited by Gianni Cesareni

Abstract Cross-talk between ERα and STAT5a was demonstrated to mediate through a direct physical association between the two proteins. By GST pull-down assays and functional assays with various constructs of ERα and STAT5a, it was shown that the C-termini of these two proteins were mainly responsible for this interaction. Furthermore, the interaction between ERα and STAT5a was demonstrated to give rise to functional changes in their signaling events. In cell transfection studies, it was shown that ERα activation could attenuate PRLR signaling through STAT5a. This ERα-mediated attenuation of PRLR signaling was substantiated by observed decreases in the phosphorylation of JAK2 and STAT5a, reduced translocation of STAT5a into the nucleus, and reduced binding of STAT5a onto a GAS-containing nucleotide. Apart from transfected cells, the interaction between ERα and STAT5a could also be observed in established breast cancer cell lines of MCF-7 and T-47D in co-immunoprecipitation studies. However, the functional consequence of the interaction in these cancer cells was very different from the transfected HEK293 cells. ER activation could lead to potentiation of PRLR signaling in MCF-7 cells but not in T-47D cells. Conversely, in both MCF-7 and T-47D cells, PRLR activation could lead to attenuation of ER signaling. These data serve to elucidate the mechanisms underlying the ERα–STAT5a cross-talk and in demonstrating that the functional consequence of this cross-talk depends on the precise milieus of the intracellular environment.

Keywords: Estrogen receptor α; STAT5a; Receptor cross-talk

1. Introduction

Both the steroid hormone estrogen and the polypeptide hormone prolactin (PRL) are important regulatory factors in mammary gland development and differentiation [1,2]. They could induce distinct signaling transduction pathways on target cells. Estrogen exerts most of its biological effects through the estrogen-specific transcription factor, the estrogen receptor (ER) [3]. The estrogen activated ERs form dimers and subsequently associate with specific consensus DNA sequences called estrogen response elements (ERE) located in the promoter regions of target genes to regulate gene transcription. ER is a member of the nuclear receptor superfamily and shares the common protein structure of this family [4,5]. The protein can be divided into several functional domains, denoted as A to F from the N terminus to the C terminus. The most conserved DNA-binding domain (DBD) C is comprised of two distinct zinc fingers and is responsible for DNA-binding and protein dimerization. The C-terminal E domain or ligand-binding domain (LBD) plays important roles in mediating ligand binding, receptor dimerization, nuclear translocation, and ligand-dependent transactivation (AF-2) of target gene expression. The N-terminal A/B domain is highly variable in both sequence and size. It usually contains a transactivation function (AF-1), which activates target genes by directly interacting with components of the core transcription machinery or with coactivators that mediate signaling to the downstream proteins. The hinge domain D contributes flexibility to the DBD versus LBD and has also, in some cases, been shown to influence the DNA-binding properties of individual receptors, thus serving as an anchor for certain co-repressor proteins. Finally, the C-terminal F domain has been shown to contribute to the transactivation capacity of the receptor. But its other functions, if any, are to a large extent unknown.

PRL induced its biological effects through the membrane bound PRL receptor (PRLR) and the Janus kinase (JAK) and signal transducers and activators of transcription (STAT) signaling cascade, mainly activating JAK2 and STAT5, including STAT5a and STA5b proteins [6,7]. Once bound to its ligand, PRLR dimerizes and activates JAK2. The activated JAK2, in turn, mediates phosphorylation of specific receptor tyrosine residues of PRLR, which then serve as docking sites for STAT5 proteins and other signaling molecules. Once recruited to the receptor, STAT5 also become phosphorylated by JAK2 on a single tyrosine residue located at the C-terminus. This is followed by the dissociation of STAT5 from PRLR and the formation of STAT5 dimers, which migrate into the nucleus to bind to γ-activated sequence element (GAS site) in the promoters of cytokine-responsive genes.

Functional interaction between steroid hormone receptors and STAT5 has been proposed [8–14]. For example, cross-talk between glucocorticoid receptor (GR) and STAT5a has been reported. GR and STAT5a have been shown to interact in both transiently transfected cells [8] and in mammary epithelial

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0014-5793/$22.00
doi:10.1016/j.febslet.2004.06.098

cells [13]. They act synergistically in inducing the STAT5a regulated β-casein promoter in transiently transfected COS cells. Recruitment of GR to the promoter by STAT5a leads to a supplementation of the STAT5a transactivation ability by the stronger GR transactivation domain [9]. Enhancement of STAT5a activity by GR might be the result of a protein interaction between GR and STAT5a, which enhance and prolong the DNA binding ability of STAT5a [11,13]. Synergy between STAT5 and progesterone receptor (PR) or mineralcorticoid receptor (MR) has also been reported [10]. In these instances, the steroid hormone receptors were reported to exert a negative influence on STAT5a transactivation through a physical interaction with STAT5a [10,14].

In the present work, functional interaction between ERα and STAT5a signaling was identified and co-immunoprecipitation was performed to confirm the binding between ERα and STAT5a. The domain(s) involved in this functional interaction in each protein was mapped using either functional assays or glutathione *S*-transferase (GST) pull-down assays. STAT5a activation events, including tyrosine phosphorylation, DNA binding and nuclear translocation were investigated in the presence or absence of ERα co-expression. In addition to the co-transfection system, two human breast cancer cell lines, MCF-7 and T-47D, which naturally express ERα and STAT5a, were used to investigate the functional interaction between ERα and STAT5a. Our data contribute to a better understanding of the mechanisms underlying ERα-STAT5a cross-talk.

2. Materials and methods

2.1. Plasmids

The pSG5-MOR mouse ERα-containing plasmid was kindly provided by Dr. Parker of Imperial College London, UK. The pXM-STAT5a plasmid was kindly provided by Dr. Hennighausen of the National Institutes of Health, USA. The pcDNA3-hPRLR plasmid containing the long form human PRLR was constructed as described [15]. For the GST fusion constructs, the DBD of STAT5a was amplified by the forward primer: 5′-ACC AGC ACG TTC ATC ATC GAG-3′ and the reverse primer: 5′-GCC CCG GTT GCT CTG TAC TT-3′. The Src-homology 2(SH2)-transcription activation domain (TAD) fragment of STAT5a was amplified by the forward primer: 5′-TTC TGG CAG TGG TTC GAC GG-3′ and the reverse primer: 5′-ACC AGC ACG TTC ATC ATC GAG-3′. Both DNA fragments were inserted in frame into the *Sma* I site of the GST expressing vector pGEX-4T-1 by blunt-end ligation. Various ERα mutants were generated by PCR (18 cycles of 92 °C for 45 s, 62 °C for 45 s and 72 °C for 2 min) using a set of mouse ERα gene specific primers (ER-F1: 5′-G GGA TCC ATG CAC ACC AAA GCC TCG-3′; ER-F2: 5′-G GGA TCC ATG TAC TGT GCC GTG TGC-3′; ER-F3: 5′-G GGA TCC ATG GTC AGT GCC TTG TT-3′; ER-R1: 5′-G GAA TTC TCA GAT CGT GTT GGG G-3′; ER-R2: 5′-G GAA TTC TCA ATG AAG GCG GTG GGC A-3′; and ER-R3: 5′-G GAA TTC TCA CAT CTG GTC AGC TGT C-3′). *Bam*H I and *Eco*R I restriction sites were introduced in the forward and reverse primers, respectively, to facilitate subsequent subcloning. The DNA fragments were inserted in frame into the eukaryotic expression vector pBK/CMV. The inserts of all the plasmids used in this study were checked by DNA sequencing to avoid mutations.

2.2. Cell culture, transfection and luciferase assays

Human embryonic kidney (HEK) 293 cells were cultured in DMEM containing 5% fetal bovine serum (FBS), 1× antibiotics P/S (100 U/ml penicillin and 100 μg/ml streptomycin). Transfection was performed using the Lipofectamine Reagent (Invitrogen) according to the manufacturer's protocol. Briefly, 2×10^5/well cells were seeded in poly-D-lysine pre-coated 24-well plate in phenol red-free MEM (Sigma) containing 1% charcoal-stripped FBS for 24 h before transfection. Luciferase (LUC) was used as the reporter gene. The reporter plasmids (ERE-LUC or β-casein-LUC, 500 ng), together with the PRLR, STAT5a or ERα expression plasmids, were co-transfected into the cultured cells as indicated in the figure legends. The water-soluble 17β-estradiol (E_2) (Sigma) (final concentration 10^{-8} M), ovine PRL (oPRL) (Sigma) (final concentration 1 μg/ml), a combination of both, or just the vehicle alone were added to the cells as indicated in the figure legends. Hormone treatment lasted for 24 h before the cells were lysed for the LUC assays.

The human breast cancer cell lines MCF-7 and T-47D were routinely kept in MEM containing 5% FBS and 1× P/S. Before use, the medium was changed to phenol red-free MEM containing 1% charcoal-stripped FBS for 48 h. Five hundred nanograms of either ERE-LUC or β-casein-LUC reporter was transfected using the same protocol. Hormone treatment was performed as indicated in the figure legends for 24 h before LUC assays.

LUC assays were performed using the Dual-Luciferase Reporter System (Promega) with the proper control normalization vector (*Renilla* luciferase).

2.3. GST pull-down assays

GST pull-down assays were performed using the Profound Pull-Down GST Protein: Protein Interaction Kit (Pierce). Expression of

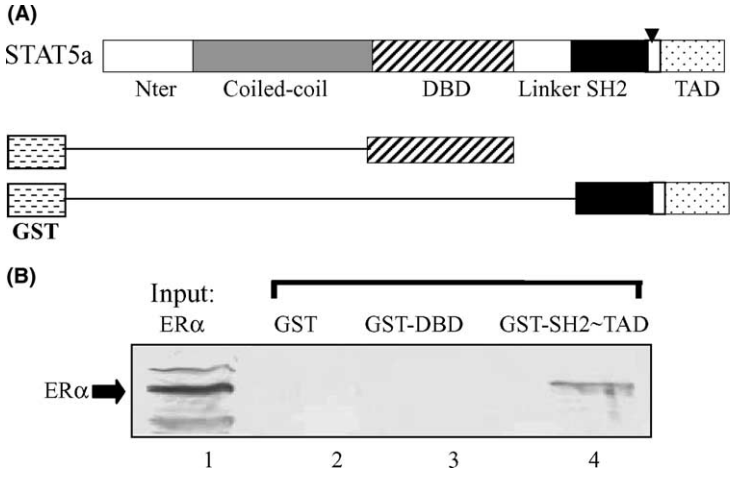

Fig. 1. ERα binds to the C-terminus of STAT5a. Panel A is a schematic representation of the two GST-fusion STAT5a mutants used in the GST pull-down assays, one containing the DBD alone and the other containing the SH2-TAD domains. The black triangle indicates the tyrosine phosphorylation position. Panel B shows the results of the various immobilized GST-fusion proteins co-incubated with lysates prepared from ERα transfected and E_2 treated HEK293 cells. Specifically bound ERα was visualized by Western blotting using an anti-ERα antibody.

GST-fusion proteins was induced by IPTG in the *E. coli* strain BL21. The induced culture (5 ml) was collected and lyzed. The bacterial expressed GST-fusion proteins were then immobilized on a glutathione gel to serve as bait proteins in the subsequent steps. Total proteins (500 μg) from HEK293 cells transfected with ERα were incubated with each immobilized GST-tagged bait protein and the bait-prey elution was then analyzed by Western blot by probing the ERα.

2.4. Whole cell extract (WCE) preparation

HEK293 cells (1×10^7/dish) in 100 mm tissue culture dish were transiently transfected with 5 μg ERα and 5 μg STAT5a expression plasmids together with 500 ng of the PRLR expression plasmid for inducing the PRL signal. Transfections using either ERα alone, or STAT5a plus PRLR were used as controls. After incubation in the transfection medium for 5 h, cells were recovered in phenol red-free MEM containing 1% charcoal-stripped FBS for 24 h. The cells were then stimulated for 30 min with 1 μg/ml oPRL, 10^{-8} M E_2, a combination of both, or just the vehicle alone. Cells were briefly washed with ice-cold 1× PBS, collected in an Eppendorf tube, and lysed in lysis buffer (20 mM HEPES, pH 7.5, 0.4 M KCl, 0.1 mM EDTA and 0.1% NP-40) supplemented with a protease inhibitor cocktail (Roche) containing 2 μg/ml aprotinin, 2 μg/ml benzamidine, 2 μg/ml soybean trypsin inhibitor, 1.5 μg/ml leupeptin, 1 mM sodium orthovanadate and 1 mM sodium molybdate. Lysates were centrifuged at $12\,000 \times g$ for 15 min at 4 °C to remove the debris and protein concentrations were measured by the BCA method (Bio-Rad). All extracts were aliquoted, frozen in liquid nitrogen, and stored at −80 °C until use. Each aliquot was thawed only once.

2.5. Co-immunoprecipitation studies

WCE (400 μg) from each sample was used in the co-immunoprecipitation studies. Immunoprecipitation was carried out at room temperature for 2 h by adding 40 μg of anti-ERα antibody (H-184) (Santa Cruz Biotechnology, Inc.) and protein G-agarose beads (Sig-

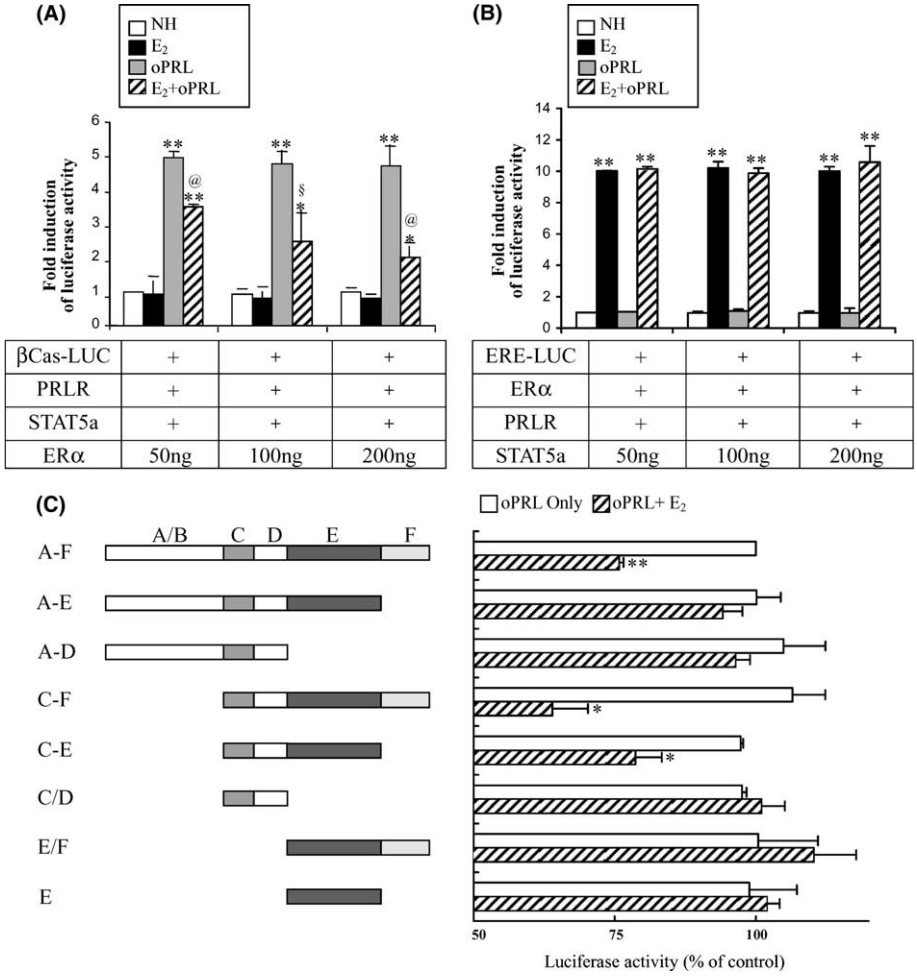

Fig. 2. ERα inhibits PRL-induced STAT5a-mediated transcription activation. HEK293 cells were co-transfected with 500 ng β-casein-LUC reporter plasmid together with expression vectors containing PRLR (50 ng), STAT5a (100 ng) and increasing amounts of ERα (50–200 ng) (Panel A). In a separate experiment, HEK293 cells were co-transfected with 500 ng ERE-LUC reporter plasmid together with 100 ng ERα, 50 ng PRLR and increasing amount of STAT5a (50–200 ng) (Panel B). The total amount of DNA used in each transfection was adjusted to 1 μg/well with the empty vector pcDNA3. The transcription responses to 10^{-8} M E_2, 1 μg/ml oPRL, or a combination of both, or just the vehicle alone (NH = no hormone control) were determined. The *Renilla* luciferase internal control plasmid was also co-transfected to correct for differences in transfection efficiency. In Panel C, HEK293 cells were co-transfected with 500 ng β-casein-LUC reporter plasmid together with expression vectors containing PRLR (50 ng), STAT5a (100 ng) and various ERα mutants (50 ng). The left hand side of Panel C shows the ERα mutants used. The transcription responses to 1 μg/ml oPRL (open bars) or a combination of 1μg/ml oPRL and 10^{-8} M E_2 (stripped bars) are shown on the right hand side of Panel C. All luciferase assays were performed in duplicates. Data are presented as means ± S.D. ($n = 6$). Statistical analysis was performed by ANOVA, followed by Tukey's multiple comparison test. Panel A: *, $P < 0.05$ compared against NH control; **, $P < 0.01$ compared against NH control; §, $P < 0.05$ comparing (E_2+oPRL) against (oPRL); @, $P < 0.01$ comparing (E_2+oPRL) against (oPRL). Panel B: **, $P < 0.01$ compared against NH control; no statistical significance was observed between (E_2+oPRL) and (oPRL) in all cases. Panel C: *, $P < 0.05$ compared against oPRL only; **, $P < 0.01$ compared against oPRL only.

ma). Precipitated STAT5a was analyzed using SDS–PAGE followed by Western blotting with an antibody probing STAT5a (L-20) (Santa Cruz Biotechnology, Inc.). For detecting tyrosine phosphorylated STAT5a and JAK2, STAT5a and JAK2 were first captured by immunoprecipitation with an anti-STAT5a antibody or an anti-JAK2 antibody (C-20) (Santa Cruz Biotechnology, Inc.). The precipitated proteins were analyzed with a phosphotyrosine specific antibody (PY20) (Santa Cruz Biotechnology, Inc.).

2.6. Electrophoretic mobility shift assays (EMSA)

An oligonucleotide encompassing the β-casein GAS site was used for EMSA. The sequence of the coding strand was as follows: 5′-TAATCATGTGGACTTCTTGGAATTAAGGGACTTTT-3′. Sense and antisense oligonucleotides were synthesized and labeled with biotin using the Biotin 3′-End DNA Labeling Kit (Pierce). Double stranded DNA was formed by annealing the single chain oligonucleotides at room temperature for at least 30 min after heating together at 80 °C for 15 min in 1× annealing buffer (20 mM Tris–Cl, pH 7.5, 10 mM $MgCl_2$ and 50 mM NaCl). EMSA was performed using the LightShift Chemiluminescent EMSA Kit (Pierce). Briefly, the total protein and the labeled probe were incubated together with 2 μl of poly (dI)-poly (dC) (1 μg/μl) and 2 μl 10× binding buffer (100 mM Tris, 500 mM KCl and 10 mM DTT, pH 7.2) in a total volume of 20 μl per reaction for 20 min at room temperature. The reaction mixtures were resolved on a 4% native polyacrylamide gel containing 0.5× TBE. Biotin-labeled DNA was detected according to the manufacturer's protocol.

2.7. Subcellular fractionation

Nuclear extracts were prepared using the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce). Cells were collected by scraping and then washed briefly with 1× PBS. After careful removal of the supernatant, the cell pellet was lyzed and layered by centrifugation at 16 000 × *g* for 5 min at 4 °C. The supernatant containing the cytoplasmic components was carefully removed. The nuclear pellet was further lyzed and cleaned by another round of centrifugation at 16 000 × *g* for 10 min at 4 °C. The nuclear extracts were stored at −80 °C in aliquots after protein determination.

3. Results

The C-terminal SH2-TAD domains of STAT5a and the N-terminal DBD were expressed as GST fusion proteins and tested for their binding abilities with ERα expressed in HEK293 cells activated with E_2. As shown in Fig. 1, only the STAT5a fragment containing the C-terminal SH2-TAD domains showed an ERα specific band after Western blot analysis, whereas the fragment containing the DBD did not bind with ERα. This thus raises the possibility that ERα may bind to STAT5a through the C-terminus of STAT5a.

This physical interaction is functionally significant, as demonstrated in Fig. 2. STAT5a activation through ligand stimulation of PRLR, as demonstrated by the β-casein-LUC reporter gene, was attenuated by ERα activation (Fig. 2A). On the other hand, however, ERα activation, as demonstrated by the ERE-LUC reporter gene, was not affected by STAT5a activation (Fig. 2B). Furthermore, using different ERα deletion mutants in the functional assays, it was found that deletion of the ERα C-terminal domains, including the F, E, and C domains, caused obvious attenuation of the inhibitory effects, while deletion of the A/B domain had no effect, indicating that the inhibition of the PRL-induced β-casein-LUC expression by E_2 could be attributed to the C-terminal C-F domains of ERα, but not the N-terminal A/B domain (Fig. 2C). Within the same assay, we found that among all the mutants, the plasmid containing C-F portion of ERα exhibited the highest inhibitory potency among all the ERα mutants. The mutant containing the C-E domains also exerted considerable inhibitory activities on STAT5a transactivation but was less effective than the C-F domains. The C domain alone or the E/F domain alone failed to inhibit reporter expression. These results indicated that the entire C-terminal C-F domains contributed to maximum inhibition of STAT5a transactivation, while the core region for such an inhibition was the C-E domains. Inclusion of the F domain increases the inhibitory activity of the core region.

Co-immunoprecipitation experiments were performed to confirm the protein–protein interaction between ERα and STAT5a expressed in HEK293 cells (Fig. 3). In these experiments, HEK293 cells were transfected with both ERα and STAT5a together with PRLR to induce the PRL signal (Fig. 3, Lanes 3–6). Transfections with either ERα alone (Fig. 3, Lane 2) or PRLR plus STAT5a (Fig. 3, Lane 1) were used as controls. Precipitation of STAT5a by ERα specific antibody was observed in conditions when ERα and STAT5a were co-expressed, regardless of the presence or absence of E_2 and/or oPRL (Fig. 3A, Lanes 3–6), indicating that the ERα–STAT5a interaction is a hormone independent event. In the absence of ERα, the STAT5a protein could not be co-immunoprecipitated (as indicated in Lane 1 of Fig. 3A). A weak STAT5a band also appeared under the condition when ERα alone was transfected (as indicated in Lane 2 of Fig. 3A). This was probably due to the endogenous expression of STAT5a protein in HEK293 cells (as indicated in Lane 2 of Fig. 3B). Western blot analyses probing STAT5a (Fig. 3B) or ERα (Fig. 3C) revealed that the observed results could not be attributed to changes in the expression levels of STAT5a and ERα.

In the JAK-STAT pathway, STAT is activated through phosphorylation by cytokine receptor-associated JAK tyrosine kinase, which is also activated by phosphorylation. Activated STAT protein then dimerizes and translocates into the nucleus to regulate target gene transcription. These STAT activation events have also been examined upon ERα–STAT5a interac-

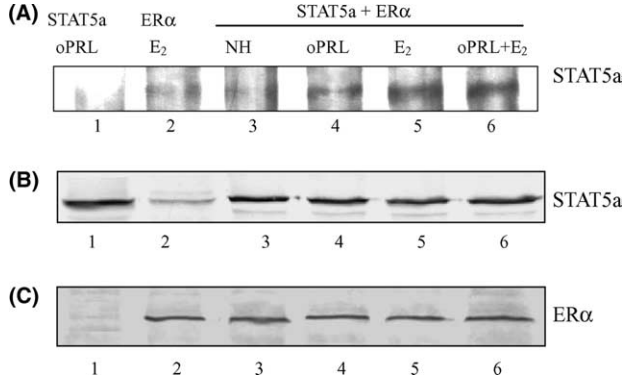

Fig. 3. Co-immunoprecipitation of STAT5a with ERα. HEK293 cells were transfected with expression vectors containing PRLR, STAT5a and ERα (Lanes 3–6), or with just PRLR and STAT5a (Lane 1), or with ERα alone (Lane 2). Cells were treated for 30 min with 1 μg/ml oPRL, or 10^{-8} M E_2, or a combination of both, or just the vehicle alone (NH = no hormone control) before lyzed for co-immunoprecipitation studies. Four hundred micrograms of total protein in each lysate were incubated with 40 μg anti-ERα antibody. The amount of co-immunoprecipitated STAT5a was analyzed by Western blotting with an anti-STAT5a antibody (Panel A). The expression levels of STAT5a (Panel B) and ERα (Panel C) in the cells were examined using 20μg of total protein from each cell lysate in the SDS–PAGE followed by Western blotting with an anti-STAT5a antibody or an anti-ERα antibody.

tion in the present work (Fig. 4). To examine these events, HEK293 cells were transfected with STAT5a and PRLR, or with STAT5a, PRLR and ERα, and then treated with oPRL alone, or with both E_2 and oPRL. By using a phosphotyrosine specific antibody, we found that STAT5a phosphorylation was significantly repressed upon ERα co-expression as compared with the no ERα control (Fig. 4A, upper panel). In the same assay, JAK2 phosphorylation level was also found to be inhibited upon ERα co-expression (Fig. 4A, lower panel). And the results were not due to the altered expression of either the STAT5a or JAK2 protein in both situations. In EMSA, an oligonucleotide encompassing the GAS site was used as the probe to assess the STAT5a DNA-binding ability. When STAT5a and ERα were co-expressed in the cultured cells, a decrease in the intensity of the PRL-induced STAT5a-DNA complex was observed as compared to the band intensity of the same complex when only STAT5a was transfected. Furthermore, nuclear extracts were prepared from these transfections and probed for STAT5a. PRL stimulation was able to trigger nuclear translocation of STAT5a (Fig. 4C, Lanes 1 and 2). An increase in the nucleus located STAT5a was also observed in cells co-expressing STAT5a and ERα treated with PRL as compared to the no hormone control (Fig. 4C, Lane 3). Further addition of E_2 caused a more obvious decrease in the nucleus located STAT5a level. Interestingly, the nucleus ERα level remained the same in cells expressing only ERα or co-expressing ERα and STAT5a (Fig. 4C, lower panel). This could be due to the fact that ER, unlike other steroid hormone receptors, is predominantly a nuclear protein regardless of whether or not it is complexed with the ligand [3,16].

Since both E_2 and PRL play crucial roles in mammary gland development, differentiation and tumorigenesis, it would be revealing to see whether the ER–STAT5 cross-talk actually takes place in cells of mammary gland origin. Thus, two human breast cancer cell lines, MCF-7 and T47D, which express ER, PRLR as well as STAT5, were employed to study such events. Direct association of ERα and STAT5a was identified in lysates from both cell lines by co-immunoprecipitation studies (Fig. 5A). In functional assays, both cells were transfected with the ERE-LUC reporter plasmid and treated with E_2 alone, or E_2 plus oPRL at different concentrations (10–100 ng/ml), to investigate whether addition of oPRL will influence the ER signaling. Data from this assay indicated that addition of PRL repressed ER signaling in both cell lines (Fig. 5B). To

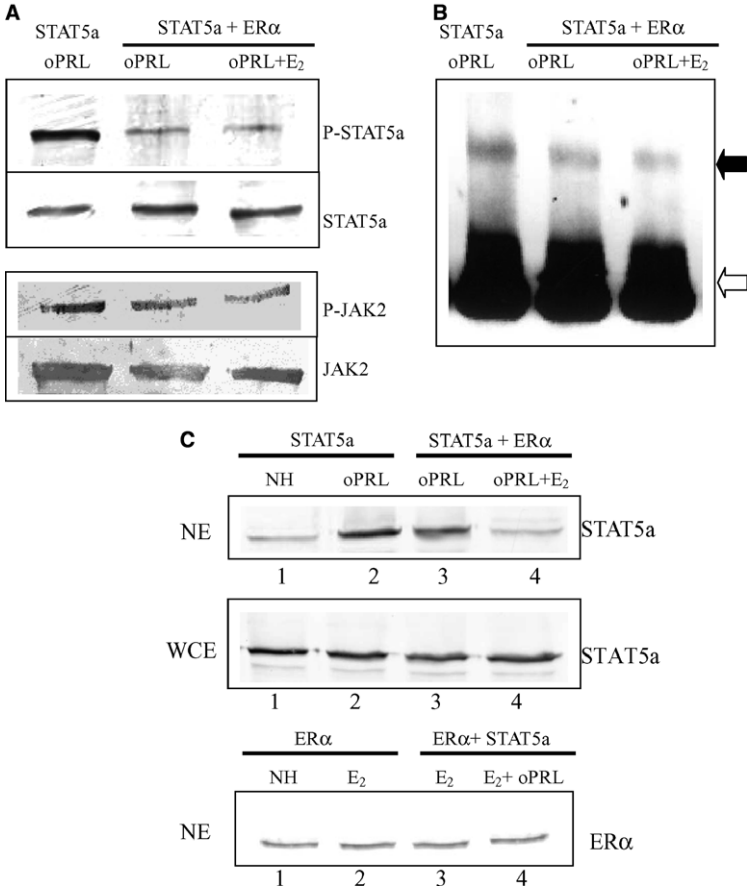

Fig. 4. ERα decreases STAT5a phosphorylation, DNA-binding and nuclear translocation. HEK293 cells were transfected with PRLR and STAT5a containing plasmids with or without ERα as indicated. Cells were then treated for 30 min with 1 μg/ml oPRL and/or 10^{-8} M E_2 as shown. NH stands for no hormone control. Panel A shows the results of the STAT5a and JAK2 phosphorylation. Cell lysates (400 μg) were immunoprecipitated with anti-STAT5a or anti-JAK2 antibodies. Western blotting was then performed with an anti-phosphotyrosine antibody. The expression levels of STAT5a and JAK2 in the cells were examined using anti-STAT5a and anti-JAK2 antibodies. Panel B shows the EMSA results of STAT5a-DNA binding. The solid arrow indicates the PRL induced complex that contains STAT5a. The open arrow indicates the free probes. Panel C shows the results of the STAT5a nuclear translocation. Nuclear fractions of transfected HEK293 cells were prepared, and 50 μg of each was separated on a 10% SDS–PAGE followed by Western blot analyses probed with anti-STAT5a and ERα antibodies.

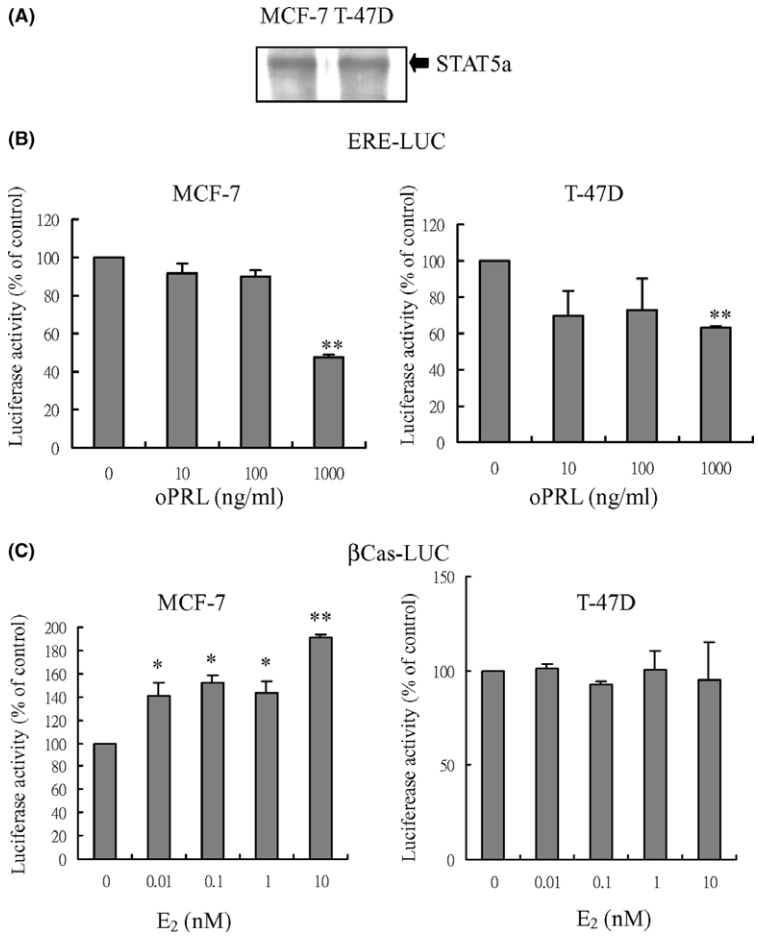

Fig. 5. ER and PRL signaling cross-talk in human breast cancer cells. MCF-7 and T-47D cell lysates were immunoprecipitated with an anti-ERα antibody and probed with an anti-STAT5a antibody (Panel A). Effects of PRL on E_2 induced transcription activity (Panel B) and effects of E_2 on PRL induced transcription activity (Panel C) were investigated in both MCF-7 and T-47D cells. The luciferase activity was expressed as a percentage relative to the control when E_2 alone (Panel B) or oPRL alone (Panel C) was used. All luciferase assays were performed in duplicates. Data are presented as means ± S.D. ($n = 6$). Statistical analysis was performed by ANOVA, followed by Tukey's multiple comparison test (*, $P < 0.05$; **, $P < 0.01$).

assess the effect of ER activation on PRL signaling, cells were transfected with the β-casein-LUC reporter plasmid and treated with oPRL alone, or oPRL plus E_2 at different concentrations (0.01–10 nM). It was observed that addition of E_2 enhanced PRL signaling in MCF-7 cells but not in T-47D cells (Fig. 5C).

4. Discussion

ER has been reported to interact with some STAT proteins [14,17,18]. In the present study, a physical association between ERα and STAT5a was clearly demonstrated in co-immunoprecipitation experiments in the transfected HEK293 cells. It is interesting to note that this protein–protein interaction was observable even in the absence of ligand stimulation. Early research on the cross-talk between GR and STAT5a also indicated that interaction between GR and STAT5a is not hormone dependent [13]. It is therefore possible that interaction between steroid hormone receptors and STAT proteins may exist widely in different tissues that express these proteins. However, enhancement of protein binding and repression of STAT5a activation in the presence of hormone were observed in this study. The role of the hormonal ligands in the cross-talk needs further investigation.

More importantly, this physical interaction between the ERα and STAT5a was demonstrated to give rise to functional changes in their signaling events. In transfected HEK293 cells, it was shown that ERα activation could attenuate PRLR signaling through STAT5a. Involvement of the TAD domain of STAT5a in the protein–protein interaction provided a direct transrepression mechanism. Functional determination of domains in ERα that are required to mediate this repression of STAT5a transactivation revealed the significance of the C to F domains in ERα. An early study on ER–STAT5 cross-talk [14] reported that the interaction relies on highly specific binding via the DBD and pointed out an indispensable role of the LBD of ER for a sufficient repression of STAT5 activity. In our experiments, the ERα mutant containing the C to E domains was demonstrated to be the shortest fragment which was able to inhibit STAT5a transactivation. Either the DBD alone or the LDB alone could not repress STAT5a activity. Our functional study further suggested that inclusion of the F domain into the core inhibitory region (C to E domains) enhanced the

repression on STAT5a transactivation. The F domain was reported to contribute to the transactivation capacity of ER, as well as receptor dimerization and co-activator interaction [19]. Thus, an integral F domain may help in LBD transrepression, or in the stability of the ERα–STAT5a complex.

This ERα-mediated attenuation of PRLR signaling was further substantiated by observed decreases in the phosphorylation of JAK2 and STAT5a, reduced translocation of STAT5a into the nucleus, as well as reduced binding of STAT5a onto the GAS-containing nucleotide. These might be subsequent to the involvement of the SH2 domain of STAT5a in the protein–protein binding. In STAT protein, SH2 domain is responsible for recruiting STAT5a to the cytokine receptor, STAT association to JAK kinase as well as the phosphorylation, dimerization, DNA-binding and nuclear translocation of STAT [20–23]. Involvement of JAK2 in receptor cross-talk recently reported that estrogen inhibits GH signaling by repressing GH-induced JAK2 phosphorylation [24]. However, a direct protein–protein interaction between ERα and JAK2 was not found in both experiments. The underlying mechanisms and the biological significance remain unknown.

In the present investigation, ERα–STAT5a binding was also identified in breast cancer cell lines but the functional outcome of such an interaction was very different from that in the HEK293 cells. Because of the biological complexities of the cells, the observed changes could result from a variety of possible factors such as the existence of ERβ, other STAT proteins, or other signaling pathways. The autocrine/paracrine nature of estrogen and PRL in MCF-7 and T-47D cells should also be taken into consideration [25–27]. Finally, co-activators or co-repressors may play important roles in the receptor cross-talk. Both ER and STAT5 may interact with additional factors called co-activators or co-repressors to mediate either activation or repression of target gene expression. Some of these cofactors are even shared in both signaling pathways. For example, a STAT cofactor known as the suppressor of cytokine signaling 2 (SOCS2) was suggested to be indispensable for inhibition of GH signaling caused by estrogen [24]. Another example is CBP/p300, which was previously suggested to serve as an integrator of multiple signal transduction pathways [28–30]. Limiting amount of this transcription co-activator was thought to result in the negative cross-talk between steroid hormone receptor and AP-1 activity, although a recent study revealed that CBP/p300 did not participate in the STAT5-mediated suppression of GR action [14]. The effects of these other cofactors in ER–STAT5 cross-talk warrant further investigations.

In conclusion, results of the present study revealed some mechanisms of steroid hormone regulation of peptide hormone action, and would have significance beyond estrogen and PRL.

Acknowledgements: We thank Dr. Parker of Imperial College London, UK in providing us the pSG5-MOR mouse ERα clone, Dr. Henninghausen of the National Institutes of Health, USA in providing us the pXM-STAT5a plasmid, and Dr. K.L. Yu of the University of Hong Kong, Hong Kong in providing us the ERE-LUC and β-casein-LUC reporter plasmids. We are also grateful to The Chinese University of Hong Kong for the provision of Direct Grants (to CHKC) and graduate studentship (to YW).

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